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Diversity of the human erythrocyte membrane sialic acids in relation with blood groups

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Abstract The composition of the human erythrocyte membrane (RBC) glycoprotein- and glycolipid-bound sialic acids of A, B, AB and O type donors was studied using a new method (Zanetta et al., Glycobiology 11 (2001) 663–676). In addition to Neu5Ac as the major compound, Kdn, Neu5,9Ac_2, Neu5,7Ac_2, Neu (de-*N*-acetylated-Neu5Ac), Neu5Ac8Me, Neu5Ac9Lt, Neu4,5Ac_2, Neu5,8$Ac_2$9Lt and Neu5Ac8S were characterised. Among these different compounds, Neu5Ac8Me, Neu5Ac9Lt, Neu4,5Ac_2, Neu5,8$Ac_2$9Lt and Neu5Ac8S have never been described and quantitatively determined before in human tissues or cells. Neu5Gc and its *O*-alkylated or *O*-acylated derivatives were not detected.

Key words: Sialic acid; Red blood cell; Blood group; Human erythrocyte

1. Introduction

The sialic acid family presents a very large heterogeneity. Besides the three different classes defined by the diversity of the substituents of the C(5) carbon atom (*N*-acetyl-neuraminic acid, *N*-glycolyl-neuraminic acid and 3-deoxy-D-glycero-D-galacto-nonulosonic acid (Kdn)), some of the hydroxyl groups can be substituted by acyl groups (*O*-acetyl, *O*-lactyl, *O*-sulphate, *O*-phosphate) or by methyl groups [1–3]. This heterogeneity is representative of a second degree of complexity of glycoconjugates. The presence of *O*-methyl groups on glycoconjugate monosaccharides was previously observed in several organisms such as 6-*O*-methyl-galactose [4] or 2-*O*-methyl-fucose [5], due to the resistance of these substituents to the classical techniques used for the cleavage of the glycosidic bonds. In contrast, the *O*-acyl groups of most constitutive monosaccharides are liberated under the conditions of glycosidic bond cleavage. This contrasts with sialic acids since their glycosidic bonds are generally labile in acidic conditions. This offered the possibility to examine the nature of these substituents in different tissues. Such determinations were generally difficult because of the necessity to perform the analysis starting from large amounts of material (for review see [1]). However, a recent method developed in our laboratory [6] overcame these problems of sensitivity since it did not require an initial purification of sialic acids after acid hydrolysis before analysis. Because of the stability of the volatile heptafluorobutyrate (HFB) derivatives of the *O*-methyl esters used for the gas chromatography (GC) mass spectrometry (MS) analysis, on the one hand, and the very specific fragmentation spectra obtained by electron impact mass spectrometry, on the other hand, it became possible to analyse sialic acids down to the picogram level.

Since the sialic acid composition of human red blood cell (RBC) membrane has not yet been reported in detail, we analysed separately the glycoprotein- and glycolipid-bound sialic acids of blood samples from individuals with A, B, AB and O blood groups. The results reveal a very large diversity of the glycoconjugate-bound sialic acids since in addition to Neu5Ac as the major constituent, the following compounds were identified: Neu5Ac8Me, Neu, Neu4,5Ac_2, Neu5,7Ac_2, Neu5,9Ac_2, Neu5Ac9Lt, Neu5,8$Ac_2$9Lt and Neu5Ac8S, as well as traces of Kdn, but the total absence of Neu5Gc.

2. Materials and methods

2.1. Chemicals

Standard Neu5Ac and Neu5Gc were purchased from Sigma (St. Louis, MO, USA). Neu4,5Ac_2, Neu5,9Ac_2 and Neu5,7,9Ac_3 were a generous gift from Prof. R. Schauer. Diazogen™ was from Aldrich (Milwaukee, WI, USA) and heptafluorobutyric anhydride (HFBAA; puriss. grade) from Fluka (Buchs, Switzerland). Heavy walled screw cap tubes (10×100 mm) and Teflon-lined caps (GL14) were from Schott (Mainz, Germany).

2.2. Isolation of human RBC membranes and delipidation procedure

For the preparation of RBC membranes, human blood was taken up on heparin from healthy individuals and washed thrice by centrifugation (for 5 min, 1000×*g* at 4°C) with phosphate-buffered saline (PBS: 25 mM sodium phosphate buffer pH 7.2 containing 150 mM sodium chloride) in order to eliminate platelets and leukocytes by pipetting the supernatant. RBC haemolysis was performed using a 10-fold dilution of PBS in cold water (39 ml per ml RBC). After vigorous agitation, the samples were centrifuged (20 min, 10000×*g* at 4°C) and the pellet was washed thrice in the lysis buffer and centrifuged in the same conditions. A last washing of the pellet was performed in cold water in order to remove salts. Preparations were considered suitable for analysis of sialic acids when the membrane pellet was white.

For delipidation of the membranes, a first extraction was carried

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Abbreviations: RBC, red blood cells; GC, gas chromatography; MS, mass spectrometry; HFBAA, heptafluorobutyric anhydride; HFB, heptafluorobutyrate; Kdn, 3-deoxy-D-glycero-D-galacto-nonulosonic acid; The abbreviations of sialic acids are after Schauer and Kamerling [1]

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out for 30 min at room temperature under vigorous agitation with a methanol/chloroform mixture (2:1, v/v) followed by centrifugation (15 min, 1200×*g*). The supernatant was recovered and the pellet was subjected to a second extraction carried out in the same conditions with a methanol/chloroform mixture (1:2, v/v). Both supernatants were pooled and evaporated under a stream of nitrogen. The dry residue constituted the glycolipid fraction and the delipidated residue the glycoprotein fraction.

2.3. Liberation and analysis of sialic acids

All operations were performed in heavy walled Teflon-lined screw caps tubes. The glycoprotein- and the lipid-bound sialic acids were analysed separately as previously described [6] with slight modifications. For the liberation of sialic acids, all samples were hydrolysed under the same conditions (105 min at 80°C in 2 M acetic acid) and cooled at 4°C. Glycoprotein samples were centrifuged (1 h, 4000×*g* at 4°C) and the supernatant was transferred into new reaction vials and lyophilised. For the glycolipid fraction, the samples were directly lyophilised.

All the samples containing the free sialic acids were subjected to a two-step derivatisation procedure: methyl esterification of the carboxyl group with diazomethane, followed by acylation of free alcohol and amino groups with HFBAA. Taking into consideration that these reagents are harmful to health, all operations have to be performed under a well-ventilated hood. The dry samples were supplemented with 200 μl of anhydrous methanol and 200 μl of a diazomethane solution in diethyl ether [6] and left for at least 4 h at room temperature after vigorous agitation. When the GC/MS analysis had to be performed, the samples were treated individually as follows. Glycoprotein samples were evaporated to dryness under a stream of nitrogen, then supplemented with 400 μl acetonitrile and 50 μl of HFBAA and heated for 5 min at 150°C in a sand bath. After cooling the samples were evaporated under a stream of nitrogen, taken up in 400 μl of acetonitrile dried on calcinated calcium chloride [6] and an aliquot of 1 μl was injected onto the Ross injector of the GC/MS apparatus. Because of the very high amount of glycoprotein-bound sialic acid in human RBC membranes, the optimal conditions of analysis should be performed starting with less than 1 mg of RBC membrane proteins. Otherwise, the quantities of reagents indicated above have to be increased proportionally to obtain a complete derivatisation and to avoid a saturation of the detector response for Neu5Ac.

For glycolipids, the samples were treated with diazomethane and HFBAA as above. Nevertheless, due to the low amount of sialic acids in the lipid extracts and to the huge amount of cholesterol (obtained as a volatile HFB derivative) overloading all GC/MS signals, a cleaning procedure of the samples was applied. After acylation with HFBAA, the samples were evaporated to dryness under a stream of nitrogen, taken up in 1 ml of dried acetonitrile and the sample was supplemented with 1 ml of heptane (or hexane) and shaken. The upper alkane phase containing the cholesterol derivative and fatty acid methyl esters was discarded. This operation was repeated once. The final remaining lower phase was re-acylated with HFBAA as described above, dried under a stream of nitrogen, taken up in dried acetonitrile and injected onto the Ross injector of the GC/MS apparatus.

2.4. Gas chromatography and mass spectrometry

The GC separation was performed on a Carlo Erba GC 8000 gas chromatograph equipped with a 25 m×0.32 mm CP-Sil5 CB Low bleed/MS capillary column, 0.25 μm film phase (Chrompack France, Les Ulis, France). The temperature of the Ross injector was 260°C and the samples were analysed using the following temperature programme: 90°C for 3 min and then 5°C/min until 260°C. The column was coupled to a Finnigan Automass II mass spectrometer (mass detection limit 1000) or, for masses larger than 1000, to a Riber 10-10H mass spectrometer (mass detection limit 2000). The analyses were performed routinely in the electron impact mode (ionisation energy 70 eV; source temperature 150°C). In order to preserve the filament of the ionisation source, the GC/MS records were performed 5 min after the injection of the sample. The quantitation of the sialic acids was performed on the chromatogram reconstituted for the ion at $m/z = 169$ specific of HFB derivatives. This procedure eliminated all interference due to different contaminants eluted in the area of the retention times of the different sialic acid derivatives.

3. Results and discussion

Consistent with the data of the literature [7,8], our study showed that the majority of sialic acids of human RBC membranes was associated with glycoproteins, the lipid-bound sialic acids always representing less than 4% of the total sialic acids. Nevertheless, the lipid-bound sialic acids varied according to the blood group: around 0.30% of the total sialic acid acids for blood groups A and B and five-fold and 10-fold higher in blood groups AB and O, respectively (Table 1). In contrast to previous studies, the GC/MS analysis of RBC sialic acid revealed a large diversity of these compounds, especially for glycoprotein-bound sialic acids.

In fact, besides the major sialic acid Neu5Ac, nine other sialic acids were detected in the glycoprotein fraction (Table

Table 1
Distribution of glycoprotein- and glycolipid-bound sialic acids in human erythrocyte membranes of A+, B+, AB+, O+ blood type donors

	Group A (%)	Group O (%)	Group B (%)	Group AB (%)
Glycolipid				
Neu5Ac	89.41 (85.3–95.8)	92.01 (88.5–97.6)	83.04 (72.3–100)	83.47 (69.6–93.4)
Neu5Ac8Me	0.00	0.00	3.4 (0–10.2)	0.00
Neu5Ac9Lt	0.00	0.00	2.29 (0–6.9)	0.00
Neu5,9Ac$_2$	10.59 (4.17–14.7)	0.82 (0–1.7)	0.00	0.00
Neu	0.00	7.17 (1.7–9.8)	11.27 (0–23.2)	16.53 (6.6–30.4)
% of total sialic acid	0.33 (0.09–0.58)	3.67 (3.56–3.80)	0.29 (0.16–0.53)	1.54 (0.39–2.20)
Glycoprotein				
Kdn	0.06 (0.04–0.07)	0.07 (0–0.19)	0.17 (0.11–0.27)	0.06 (0.00–0.13)
Neu5Ac	86.76 (83.39–90.27)	84.53 (79.77–87.66)	85.66 (83.19–87.74)	78.26 (76.43–80.08)
Neu5Ac8Me	1.6 (0.23–3.06)	2.36 (2.00–3.21)	2.55 (2.25–3.00)	1.67 (1.18–2.16)
Neu5Ac9Lt	1.2 (0.22–2.24)	4.12 (1.97–5.05)	2.86 (2.07–3.45)	8.14 (5.83–10.44)
Neu5,9Ac$_2$	0.34 (0.23–0.50)	0.31 (0.21–1.23)	0.23 (0.17–0.29)	0.14 (0.13–0.15)
Neu4,5Ac$_2$	0.53 (0.44–0.66)	0.50 (0.24–1.01)	0.19 (0.00–0.37)	0.33 (0.29–0.37)
Neu	3.88 (2.81–5.20)	4.44 (3.25–7.64)	3.72 (3.25–4.63)	5.21 (4.71–5.70)
Neu5,7Ac$_2$	0.34 (0.16–0.60)	0.24 (0.13–0.36)	0.18 (0.13–0.23)	0.17 (0.16–0.17)
Neu4,5Ac$_2$9Lt	3.98 (1.45–5.34)	2.23 (0.51–4.64)	2.93 (2.46–3.70)	3.94 (2.10–5.79)
Neu5Ac8S	1.30 (1.13–1.58)	0.65 (0.52–0.79)	1.02 (0.45–1.16)	0.93 (0.85–1.01)
KdnAcx	absent	0.54 (0.00–1.25)	0.49 (0.29–0.74)	1.16 (0.00–2.33)
Neu5Gc	absent	absent	absent	absent
% of total sialic acid	99.67 (99.91–99.42)	96.33 (96.44–96.20)	99.71 (99.84–99.47)	98.46 (99.61–97.80)

Data were obtained from three different donors in each blood group and are expressed as mean and experimental variation.

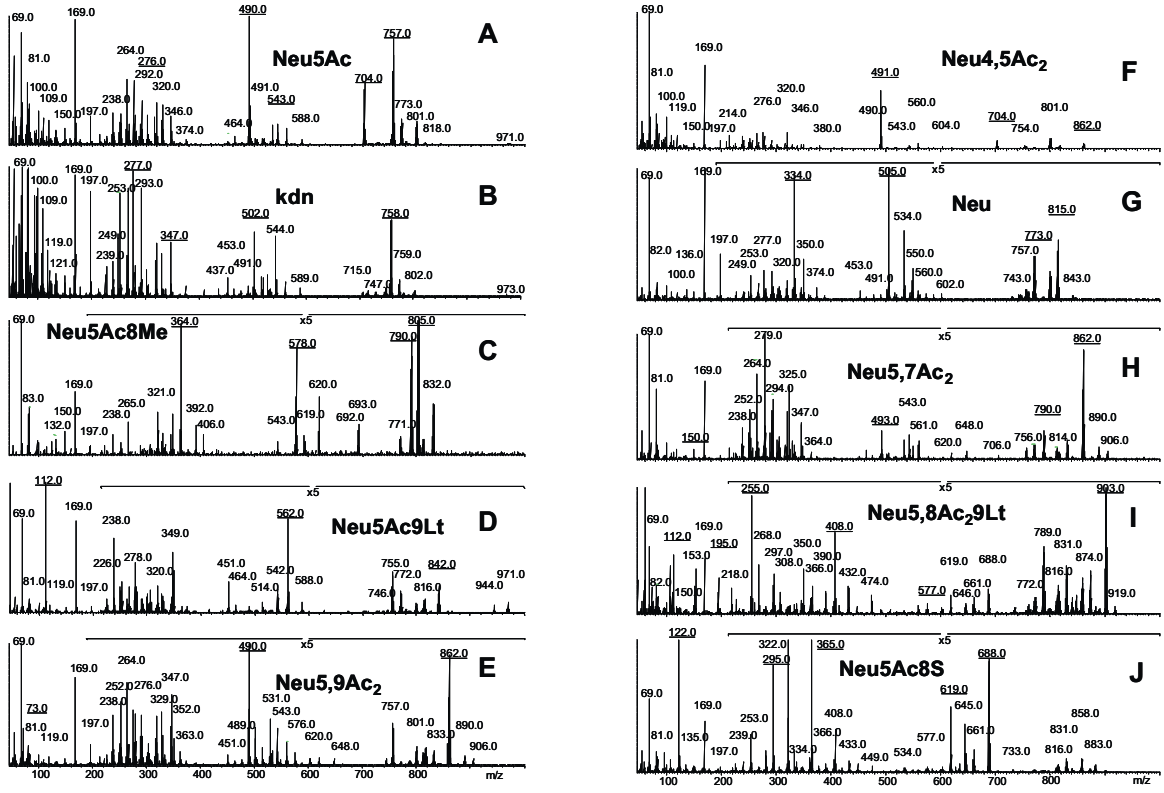

Fig. 1. Electron impact mass spectra of the HFB derivatives of the methyl esters of sialic acids: (A) Neu5Ac; (B) Kdn; (C) Neu5Ac8Me; (D) Neu5Ac9Lt; (E) Neu5,9Ac$_2$; (F) Neu4,5Ac$_2$; (G) Neu; (H) Neu5,7Ac$_2$; (I) Neu5,8Ac$_2$9Lt; (J) Neu5Ac8S (all the spectra shown here were obtained on human RBC material). In C, D, E, G, H and I the bars with '×5' correspond to a five-fold amplification of the signal. Reporter ions important for the identification of the different compounds are underlined. Note in A and B that common ions to Neu5Ac and Kdn differ from 1 atomic mass unit.

1) in the order of their GC retention times: Kdn, Neu5Ac8Me, Neu5Ac9Lt, Neu5,9Ac$_2$, Neu4,5Ac$_2$, Neu, Neu5,7Ac$_2$, Neu5,8Ac$_2$9Lt and Neu5Ac8S. In addition, a di-*O*-acetylated Kdn was detected.

Kdn was unambiguously identified since this compound showed a very typical GC elution profile, since it was the first compound of the sialic acid series to be eluted on classical methyl-siloxane liquid phases [6], its anomers being eluted in an area of the GC/MS chromatogram relatively free of contaminants so that a very low amount of this compound could be unambiguously identified by its fragmentation pattern (Fig. 1B). This observation confirmed the finding of Inoue et al. [9], reporting the presence of Kdn in human RBC. In these membranes, Kdn represented less than 0.2% of Neu5Ac and was undetectable in one sample of the B group and one sample of the AB group. The presence of a relatively more abundant compound of the Kdn family was detected (relative retention time to Neu5Ac = 1.138), showing fragmentation ions characteristic of a di-*O*-acetylated Kdn derivative [6]. However, since compounds with identical fragmentation patterns to those identified in the previous study [6] were not found in our analyses, the position of the *O*-acetyl groups could not be determined.

Neu5Ac8Me, which was identified for the first time in mammalian tissues using our method [6], was characterised by its retention time and its specific ions at $m/z = 578$ and 805 (Fig. 1C). Its presence in human RBC was verified in samples subjected to acid-catalysed methanolysis, followed by GC/MS analysis of HFB derivatives [10], since this compound behaves differently from the derivative of Neu5Ac due to the stability of the 8-*O*-methyl group to methanolysis. This compound was found at higher levels in the RBC of the AB group in which it represented up to 10% in one individual.

A second not yet identified sialic acid in human was Neu5Ac9Lt, a compound characterised by an intense ion at $m/z = 112$, which is characteristic of 9-*O*-lactylated sialic acids [6] and by a specific fragmentation pattern (Fig. 1D). This compound was particularly abundant in RBC of the O and AB groups in which it represented more than 10% of the total sialic acids in one of the samples (Table 1).

Neu5,9Ac$_2$ was the only *O*-acetylated sialic acid already described in human RBC [11–13], evidenced by specific lectins [14–18], but quantitative data on its amount in human RBC had not been determined before. Using GC/MS analysis, this compound was characterised by a series of specific ions at $m/z = 862$, 490 and 73 (Fig. 1E). In all our analyses, this sialic acid was a very minor compound, always representing less than 1.5% of the total sialic acids.

Using our method, Neu4,5Ac$_2$ was for the first time identified in human tissue or cells. In fact, it was detected at a similar level as Neu5,9Ac$_2$ in all human RBC samples so far analysed, except in one of the B group, and was characterised by its retention time and specific ions at $m/z = 861$ and 491 (Fig. 1F).

Neu (*N*-de-acetylated Neu5Ac) was also for the first time reported to be present in all RBC samples. It was unambiguously identified by its retention time and specific ions at $m/z = 505$ and 815 (Fig. 1G). The presence of this compound was

not due to a methodological artefact [6], since it was always found only in specific samples and since its level in commercially available Neu5Ac standard subjected to the entire procedure of hydrolysis and derivatisation was less than 0.01% of Neu5Ac. Furthermore, the presence of this compound in human tissues was indirectly presumed using specific antibodies and by the loss of immunoreactivity upon *N*-acetylation [19]. In addition, it was demonstrated that this compound was over-expressed in some malignant tumours [19–21], a point also supported by our recent studies (Zanetta et al., in preparation). Its higher relative abundance in the AB group glycolipid fraction was not observed in the glycoprotein fraction.

Neu5,7Ac$_2$, which was characterised by its retention time and its fragmentation pattern with ions at $m/z = 150$, 494, 790 and 862 (Fig. 1H), has never been reported before in human RBC. It was detected in all samples at a very low level.

A relatively abundant minor sialic acid, Neu5,8Ac$_2$9Lt, was actually found in all RBC samples, but more abundant in RBC of the AB group. This compound was easily identified by its ion at $m/z = 112$ and intermediate intensity ions at $m/z = 195$ and 255 (Fig. 1I). No other mono-*O*-acetylated-9-*O*-lactylated compounds could be detected.

Neu5Ac8S was only recently identified in mammalian tissues [6], but the present article demonstrated for the first time its presence in humans, although it was present at a low level in all RBC membranes. This compound was easily detected by its retention time and its characteristic fragmentation pattern including a series of specific ions at $m/z = 122$, 295 and 365 ([6]; Fig. 1J). It always represented more than 0.5% of the total sialic acids.

Because of contradictory data in the literature, particular attention is drawn to Neu5Gc as a constituent of RBC membranes. Our study clearly excluded its presence in human RBC. In our GC/MS system, Neu5Gc has a specific retention time and a specific fragmentation pattern [6] and quantities of this compound as low as 10^{-9} relative to Neu5Ac could be detected. Nevertheless, we never found any trace suggesting the presence of this compound. Therefore, these data obtained on RBC contradicted some previous reports [22] suggesting the presence of Neu5Gc derivatives in these membranes. Based on the examination of the precise biosynthetic pathways of sialic acids, it was proposed that human tissues could not synthesise Neu5Gc [23,24]. This view was also supported by the fact that multiple analyses of normal and cancerous human tissues did not allow the detection of traces of Neu5Gc. Previous data suggesting the presence of Neu5Gc in some tumour-specific antigens could be due to the presence of another sialic acid derivative, the 1,7 intramolecular lactone of Neu5Ac [6], a compound very stable in acidic or alkaline media and likely at the origin of this confusion, as discussed elsewhere [6]. This lactone was totally absent from the RBC samples analysed in this study, but was present (sometimes at very high level in some malignant tumours).

In the domain of lipid-bound sialic acids, besides Neu5Ac, which is always the major compound, only Neu5Ac8Me, Neu5Ac9Lt, Neu5,9Ac$_2$ and Neu were detected at different levels (Table 1). Neu5Ac8Me was only detected in RBC of two individuals of the B group and absent from the other samples, as was Neu5Ac9Lt. In contrast, Neu5,9Ac$_2$ was only present at a relatively high level in all the individuals of the A group (and one of the O group at a much lower level), whereas it was not detectable in the other samples. Neu was never detected in the lipid-bound sialic acids in the A group, but was found in all individuals of the AB group and at a lower level in all individuals of the O group (Table 1). Because of the relative heterogeneity of the data, it was difficult to conclude to an association of specific sialic acids with blood groups, the heterogeneity likely representing an individual specificity. Nevertheless, we could suggest that the presence of lipid-bound Neu5,9Ac$_2$ was more associated with the A group, Neu with the AB group and Neu5Ac9Lt with the B group.

4. Conclusions and perspectives

This GC/MS study shows for the first time a very high heterogeneity of the sialic acids in human RBC membranes, especially in the glycoprotein fraction, leading to the identification of 10 sialic acids. It confirms previous studies indicating that besides the major sialic acid Neu5Ac, two other sialic acids (Kdn and Neu5,9Ac$_2$) are present in these membranes and were unambiguously identified and quantified using an appropriate physico-chemical technique [6]. The existence of de-*N*-acylated sialic acid (Neu), suggested by indirect methods, was also verified. Six other different sialic acids, which had not been identified before in human tissues, could be unambiguously demonstrated in human RBC membranes, i.e. Neu5Ac8Me, Neu5Ac9Lt, Neu4,5Ac$_2$, Neu5,7Ac$_2$, Neu5,8Ac$_2$9Lt and Neu5Ac8S at significant levels, but the presence of Neu5Gc is excluded. This study also shows that the population of glycoprotein-linked sialic acids in human RBC membranes is more complex than that of the glycolipid-bound sialic acids. Despite the importance of the variations in the sialic acids of RBC membranes of the different individuals of the same blood group, this study shows a quite specific association of Neu5,9Ac$_2$ with glycolipids of the A group, of Neu with glycolipids of the AB group and of Neu5Ac9Lt with glycoproteins of the AB group, the reason for these associations remaining obscure. The presence of these new compounds gives rise to fundamental questions on the biological significance of the diversity of the sialic acid in human tissues, their biosynthetic and degradation pathways and their involvement in the normal and pathological physiology of humans.

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